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II. AGAR ANAPHYLATOXIN: GUINEA-PIG SERUM

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SYNOPSIS

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SUMMARY

The previous study on trypanosome anaphylatoxin showed clearly that the serum was the real source of the poison, and that the organisms merely played a part common to all so-called in-vitro antigens. And, since the collection of the trypanosomes was a laborious as well as expensive procedure, it was desirable to employ a more convenient reagent for the further prosecution of the study. The agar solution of Bordet[1] proved to be a most useful reagent and was employed, therefore, in the greater part of the work that followed. The serum of choice was that of the rat, on account of the ease with which it gives rise to a very active anaphylatoxin; but the sera of the rabbit and guinea-pig were utilized, from time to time, to confirm and extend the observations made with rat serum.

Bordet prepared his solution as follows: 0.5 gm. of agar was dissolved in 100 c.c. of 0.75% salt solution and this then was tubed and sterilized. The next day the gel was broken up by thorough shaking, and 5 volumes of fresh guinea-pig serum were added to 1 volume of the agar. The mixture (0.2 + 1), after being kept for 2 hours at 37 C. was centrifugated. The clear liquid when injected intravenously into guinea-pigs in dose of 4 to 5 c.c. gave the characteristic symptoms of anaphylactic poisoning with death in a few minutes. These results were soon confirmed; among others, by Nathan[2] and by Tchernoroutzky,[3] who found that the serum could be toxified by one-tenth the amount of agar employed by Bordet, that is, 0.02 c.c. of agar (= 0.1 mg. agar) per cubic centimeter of serum. It will be shown that considerably less than the amount mentioned will produce anaphylatoxin, especially with rat serum.

Inconstancy in results was a striking feature in the work of others, and such was likewise found to be the case in this study. For that reason, an enormous amount of time was devoted to ascertaining the optimal conditions necessary to the maximal and speediest production of poison. The understanding of a reaction is possible when the result of chance is replaced by one of certainty. Most of this explorative work was done with rat serum, and a considerable portion of it with guinea-pig serum. It may be stated here that eventually it became possible to toxify rat serum in less than 15 minutes, and to such an extent that 0.25 c.c. caused acute death.

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[1] Compt. rend. Soc. de biol., 1913, 74, pp. 225, 877.
[2] Ztschr. f. Immunitätsf., 1913, 17, p. 478.
[3] Compt. rend. Soc. de biol., 1913, 74, p. 1213.

Jobling and Petersen,[4] by the action of chloroform on guinea-pig serum, obtained a toxic serum which was fatal to a 210-gm. guinea-pig in dose of 0.31 c.c. Bronfenbrenner,[5] by bringing together placenta and pregnant guinea-pig serum, secured an anaphylatoxin, the lethal dose of which was 0.5 c.c. per 260 gm. of guinea-pig. In view of these results, it seemed that it should be feasible, by means of agar, to toxify guinea-pig serum to the same degree. This, however, was not accomplished, tho on a number of occasions the lethal dose was reduced to 1 c.c. and even approximated 0.75 c.c. Without doubt, further effort will give an even more toxic serum. As the result stands, it means that the guinea-pig serum possesses some inhibiting factor which does not exist in that of the rat. Strikingly suggestive is the fact that the guinea-pig, which is looked upon as being extremely sensitive to anaphylatoxin, should yield a relatively difficultly toxifiable serum, whereas the insusceptible rat gives a serum which can be rendered toxic with the greatest ease.

The best method of treating serum with agar which has been devised is liable to give somewhat inconstant results. Tchernoroutzky believed that the serum of young guinea-pigs, weighing from 400 to 500 gm., became more poisonous than that from the adult. He placed the lethal dose of the anaphylatoxin from the former at 1.2 c.c. per 100 gm. (ranging from 0.5 to 2 c.c.); while for that having the latter origin, the dose was from 2 to 3 c.c. (ranging from 1.5 to 4.3 c.c.). It has not been possible to confirm this view; the serum from adult guinea-pigs can yield as active a poison as that from the relatively young. Irregularity in results occurs in both kinds of sera. Trifling details in manipulation often give considerable variations in results.

The behavior of pregnant serum with placenta, as noted by Bronfenbrenner, might suggest that it was more labile than that of the male, but tests that were made with this in mind, failed to show any difference. Thus, by the sol-gel method, which will be considered later, a mixture of sol and pregnant serum (in the ratio of 0.1: 1), after being iced for 18 hours and then kept at 37 C. for 21 hours, caused acute death in dose of 3 c.c., while a dose of 2 c.c. was without effect. In other tests, the mixture of gel and pregnant or nonpregnant serum (0.25: 1) was incubated for 1 hour, then centrifugated at 3000 revolutions, after which it was again placed at 37 C. for 3 and 35 hours, and tested; the treated sera in dose of 2 c.c. were without effect.

Another suggested explanation of the irregularity in anaphylatoxin-production deals with the age of the serum, it being supposed that a serum which has been kept for a day or more is less toxifiable than a fresh one. This view cannot account for the inconstant results in these studies since the sera were usually employed immediately after centrifugation, or at most 2 or 3 hours later. Moreover, it is a fact that sera which have been iced for a day can be toxified. Thus, Exper. 1 of Table 19 shows the production of poison in a 36-hour serum. Like results have been obtained with rabbit sera, and in the case of rat serum kept for 1 day there is no difficulty in producing the maximal toxicity of a 0.25-c.c. lethal dose.

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[4] Jour. Exper. Med., 1914, 19, p. 485.

[5] Ibid., 1915, 21, p. 480.

A review of the results obtained, not only with guinea-pig serum but also with that of the rat and rabbit, shows clearly that the sera of a given species are subject to variation, and that no perfect regularity in toxifying with agar can be expected. Wherein the individuality of the serum rests cannot be indicated at this time. It certainly is not directly associated with the presence of lipoids, since repeatedly it was found that a white milky serum was as readily toxifiable as one that was water-clear. Tests made with fasting sera likewise gave results not unlike those with ordinary sera.

As regards the speed of anaphylatoxin-production in guinea-pig serum, under the influence of agar, it may be said that with proper conditions a minute or two suffices to render a large dose of serum acutely fatal. No special effort was made to establish this point, but in one instance (Table 30, No. 1) a dose of 3 c.c. was fatal within 8 minutes after the agar-serum mixture was made. In another instance, 3 c.c. was fatal after incubation for 10 minutes. Indeed, this dose tested after incubation at 37 C. for 15 minutes is regularly toxic. Tests made on 5 different days, the incubation being 30 minutes, gave toxic sera such that 1.5 c.c. was acutely fatal. Similarly, in 3 experiments, 1 c.c. was found to be the lethal dose after an incubation of from 1½ to 2 hours.

The agar solution employed in these studies was prepared by the addition of 100 c.c. of distilled water to 0.5 gm. of ordinary thread agar. The mixture, in a plugged Erlenmeyer flask of 150-c.c. capacity, was usually sterilized by heating in an autoclave at 120 C. for 5 minutes. Sterilization at 100, 110, 120, or 130 C. seems to have no effect on the toxifying power of the agar, and yet it is possible that the hydrolytic cleavage, by prolonged heating at higher temperatures, may affect the physical state thereby altering the inducing power. Nathan,[6] it may be mentioned, obtained about the same result with starch boiled for 2 hours or one-half minute. On the other hand, with inulin[7] he found that while the suspension toxified, the solution obtained by warming to 68 C. for 2 minutes was without effect.

The agar prepared as here described, on cooling solidified to a solid gel. This was measured out for tests by means of a cut-off full-bore pipet, either as a solid, or a semigel, after thorough shaking of the solid mass. The solidity of a gel will vary with the extent of the supporting sides of the container. Thus, in a 150-c.c. Erlenmeyer flask the agar will form a gel so solid that its surface will not show the slightest tendency to drop when the flask is placed horizontally; on the other hand, in a 500-c.c. flask the same amount of agar will form only a thin layer on the bottom which will not hold up when the flask is tilted. As a rule the agar was transferred to a smaller flask (50-c.c.), which was kept nearly full. Before use, the agar was always liquefied by heating in a water bath at 100 C. for 15 minutes. It was then placed either in a Roux water bath at 37 C., or at 50 C., for 2 hours to form a sol; or in cracked ice for 1 or 2 hours to form a hard gel.

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[6] Ztschr. f. Immunitätsf., 1913, 18, p. 646.

[7] Nathan, ibid., 1914, 23, p. 209.

The consistency of an agar undoubtedly exerts a considerable influence on its action as a toxifying agent. One ideal condition is that the agar should be distributed in the serum as homogeneously and in as fine a state of division as possible. Obviously, this condition is hardly realized when the solid or semisolid gel is added directly to the serum. On the whole, better and more consistent results were obtained when the agar was measured out as a sol. As will be shown, the sol-serum mixture when incubated at once does not give as good results as when incubated after a period of icing.

The mixture of agar and normal guinea-pig serum yields a fair precipitate on incubation, a fact recognized by Bordet. He also noted that this precipitate did not occur in serum which is inactivated at 56 C. In normal rabbit serum the separation is less marked, while in the rat serum it is decided. With rat serum this is considerably decreased when the serum is heated to 50 C. for half an hour. The quantity of precipitate which forms is a fair index of the capacity of the serum to yield anaphylatoxin. The precipitate is not to be interpreted as due to an adsorption process whereby a preformed poison is liberated or unmasked; or to one whereby a ferment is uncovered and allowed to act on the serum proteins, thus giving rise to poisonous cleavage products.

For other details of the technic employed reference must be made to Part I.

EXPERIMENTS WITH AGAR SOL AT 50 C.

The tests with agar sol were made with the expectation that the greater fluidity would give an extreme dispersion of the agar throughout the serum, thereby securing a maximal toxifying power. In these and similar tests with other sera it is to be borne in mind that the agar remains dispersed as a sol. The relatively feeble action of the dispersed sol as contrasted with that of the gel will be apparent when comparison is made with the subsequent work.

The results as seen from Table 19 were not particularly encouraging. The irregularity in the poison-production was especially perplexing, and the cause was not understood until later. The chief fallacy in this and much of the early work lay in the failure to make serial injections, at short intervals, immediately after mixing the sol and serum. Moreover, the individual resistance of the recipient must also be remembered. The real value, of course, lies in the positive tests which indicated the presence of a lethal dose of the poison; the failure to kill must not be given too rigid an interpretation.

For the 9 experiments recorded in Table 19, a 50 C. sol was prepared in the manner indicated heretofore. This was added to guinea-pig serum, previously warmed to 37 C., in the ratio of 1:4, the amounts used being usually 3 and 12 c.c.

respectively. The mixture was made in a small Erlenmeyer flask, and to secure thorough distribution it was swung for a definite time. This was 1 minute for Expers. 1 to 6; 2 minutes for Exper. 9, and ¼ and ½ minute respectively for Expers. 7 and 8. Each mixture was then centrifugated at 3000 revolutions for 5 minutes as soon as made, or after a short incubation at 37 C. (Expers. 3 to 6). In a few tests the centrifugated serum was injected at once (Nos. 1, 13, 15, 19); in these cases the interval between mixing and injection was about 10 minutes. In all the other tests, the treated centrifugated serum was incubated for 2 hours or more, at 37 C. The amount of serum actually injected is given in the table, allowance being made for the increase in volume due to the water left after centrifugation.

The serum employed for Exper. 1 had been iced for nearly 36 hours. It will be seen that this old serum gave as good results as the fresh sera. A pooled serum was used for Expers. 2 to 6, another pool served for Expers. 7 and 8, and still another for Exper. 9. The same agar, on different days, was used for Expers. 1 to 6; it was sterilized at 120 C., while that used for Expers. 7 to 9 was heated at 130 C.

It will be seen that, under the conditions given, a toxic dose of 3 c.c. was obtained in 6 of 9 experiments, usually after incubation for 2 hours. In one test, 1.7 c.c. proved fatal.

TABLE 19

ACTION OF AGAR SOL (50 C.) ON GUINEA-PIG SERUM (37 C.) (RATIO 0.25:1)

Exper.	Guinea-Pig		Serum*			Result
	No.	Weight	B. C. at 37 C.	A. C. at 37 C. (hr.)	c.c. (intravenously)	
I	1	195	—	—	3.0	Very slight
	2	190	—	2½	“	2′45″
II	3	170	—	2	“	3′50″
	4	170	—	20½	1.7	Very slight
III	5	172	2′	2	3.0	Good
	6	225	“	6	“	Slight
IV	7	170	5′	2	“	Fair
	8	172	“	5	“	4′45″
V	9	170	10′	2	“	Fair
	10	185	“	6	“	2′50″
VI	11	175	15′	2	“	Fair
	12	225	“	6	“	Fair
VII	13	175	—	—	“	Slight
	14	195	—	2	“	Fair
VIII	15	200	—	—	“	Very slight
	16	202	—	2	“	3′20″
	17	176	—	“	1.7	3′
	18	170	—	“	0.85	Slight
IX	19	230	—	—	3.0	11 hr.
	20	170	—	2	“	4′25″
	21	175	—	“	1.7	Very slight

* The dash indicates that the mixture was centrifugated as soon as made. B. C. and A. C. mean before and after centrifugation, respectively.

The 10 experiments given in Table 20 differ from the preceding (Table 19), in that the serum was chilled to 0 C. before addition of the sol. The mixtures were swung for 1 minute and then placed in cracked ice for varying periods of time, after which they were centrifugated; the resulting treated serum was then placed at 37 C. for 2 hours and injected. In these tests 2 pooled sera were used, one for Expers. 3 to 8, and the other for the remaining. The same agar was used for all the trials.

TABLE 20

ACTION OF AGAR SOL (50 C.) ON GUINEA-PIG SERUM (0 C.) (RATIO 0.25:1. MIXTURE KEPT AT 0 C., THEN CENTRIFUGATED AND INCUBATED)

Exper.	Guinea-Pig		Serum			Result
	No.	Weight	B. C. at 0 C.	A. C. at 37 C. (hr.)	c.c. (intravenously)	
I	1	197	—	2	3	5′10″
II	2	220	1′	"	"	Near-kill
	3	171	1′	"	"	Slight
III	4	200	2.5′	"	"	3′40″
	5	170	2.5′	"	2	Severe
IV	6	170	5′	"	3	Fair
V	7	207	10′	"	"	Slight
VI	8	215	15′	"	"	Very slight
VII	9	195	30′	"	"	Near-kill
VIII	10	190	1 hr.	"	"	Near-kill
IX	11	187	2 hr.	"	"	Fair
X	12	170	3	"	"	Very slight

The results obtained in this series of tests were decidedly inferior to those of the series recorded in Table 19; in only 2 of 10 experiments was the serum rendered toxic so that the lethal dose was 3 c.c., tho 3 tests gave 'near-kills.' An essential condition, not provided in this series, is incubation at 37 C. prior to centrifugation. The effect of such incubation is indicated in Table 19; in the experiments there recorded the sol-serum mixture had an initial temperature of 37 C. or better (see also Table 22).

The series of tests was made to see the effect of icing for variable periods of time, and it would seem from the table as if the short period gave the better result. Here again is a fallacy of interpretation, since extremely severe shocks were obtained in Tests 2, 9, and 10. Had duplicate tests been made, that is, 2 guinea-pigs inoculated at the same time with the same material, the number of deaths without doubt would have been increased.

The conclusion which can be drawn from these two sets of experiments is that the 50 C. sol can be used to toxify serum, but the results are not so good as with other methods. Further, incubation of the sol-serum mixture is quite essential. A 37 C. sol was not tried with guinea-pig serum since a greater effect was expected with that of 50 C. As it is, the lethal dose of 3 c.c. represents the toxifying power under these conditions.

EXPERIMENTS WITH AGAR GEL, 0 C.

In the endeavor to ascertain the best conditions for anaphylatoxin-production, many experiments were made with solidified agar or gel.

With the object of having this in as definite a state as possible, the agar for each experiment was first liquefied in a water bath, at 100 C. for 15 minutes, and then placed in cracked ice at 0 C. for 1 hour. Before use, this hard gel was broken up by vigorous shaking for about 20 seconds and the semisolid mass was then measured out by means of a full-bore pipet. In other tests, this cut-off pipet was plunged into the solid gel to the desired depth, and the cylinder of definite volume was then transferred to the serum flask.

The serum employed in these tests was also iced for at least half an hour. After the addition of the gel, the flask was vigorously swung for about 2 minutes in order to break up the agar as finely as possible. A homogeneous mixture under these conditions is out of the question; an examination will always show the agar to be in lumps of varying size, up to 2-3 mm. in diameter.

The mixtures of gel and serum, both at 0 C., were then treated in various ways. For the experiments given in Table 21, the mixture was kept at 0 C. for a given length of time, after which it was centrifugated at 3000 revolutions; in Expers. 2, 3, and 4, it was kept for 2, 6, and 12 minutes respectively; in the others, 15 minutes. The same agar and a pooled serum were used for the 3 experiments mentioned; for Expers. 1 and 5 another pooled serum and agar were used. The clear serum was then tested for its toxicity either at once, or after incubation at 37 C. for the stated time.

The results it will be noted were rather poor since but 2 fatal shocks were obtained in the 6 experiments. As in the case of the results given in Table 20, this failure must be ascribed in part to the lack of incubation of the gel-serum at 37 C. prior to the centrifugation; and, in part, to the infrequent testing. The reaction induced by the agar occurs best at higher temperatures. If, therefore, the agar is removed by centrifugation before this reaction takes place, obviously no toxicity other than the primary should be observed. However, complete removal of the agar does not occur with ordinary centrifugation and hence some reaction occurs on subsequent incubation, as will be seen from this and other tables.

In another series of experiments, similar to that just detailed, the gel-serum mixtures were kept at 0 C. for 6, 12, 24, 36, and 72 hours, respectively. Each mixture was then centrifugated at 3000 revolutions for 4 minutes, incubated, and tested at varying intervals from 1 to 32 hours. The test dose was 1.5 c.c. or less in all these experiments. Three typical acute deaths were obtained; one of these occurred with the mixture which had been iced for 6 hours and then, after centrifugation, incubated for 12 hours; with the set iced for 36 hours, one death resulted at the 6-hour test, and one at the 9-hour test, the latter being due to 1.25 c.c. of serum. Three other guinea-pigs showed severe effects but in the majority (19 of 25) the result was slight or nil. It may be added that in one other experiment similar to this one, the gel-serum mixture being iced for 21 hours, centrifugated, and then incubated for 14 hours, a fatal acute shock was obtained with 1.25 c.c.

TABLE 21

ACTION OF AGAR GEL (0 C.) ON GUINEA-PIG SERUM (0 C.); (RATIO 0.25:1. MIXTURE KEPT AT 0 C., THEN CENTRIFUGATED AND INCUBATED)

Exper.	Guinea-Pig		Serum			Result
	No.	Weight	B. C. at 0 C. (hr.)	A. C. at 37 C. (hr.)	c.c. (intravenously)	
I	1	178	½	—	3	Very slight
	2	170	½	6½	"	Slight
II	3	180	1	3½	"	Very severe
	4	198	"	9	"	Very slight
III	5	170	"	3½	"	3′50″
	6	180	"	9	"	Slight
IV	7	186	"	3½	"	Very slight
	8	185	"	9	"	Slight
V	9	185	1½	—	"	Very slight
	10	205	1½	5½	"	Slight
VI	11	210	3	—	"	Very slight
	12	170	3	6	"	3′30″

These results, as pointed out, are to be ascribed to the minute amounts of residual agar left in the serum after centrifugation. It is always possible, however, that the mere incubation of normal guinea-pig serum extending over a long period of time, with tests at frequent intervals, might show increased primary toxicity. Such increase has been frequently met with in the case of rat serum.

An attempt at the complete removal of the agar was made in the following experiments. In these the gel-serum mixtures were kept at

0 C. for 1, 3, 6, 9, 12, 18, and 24 hours, then centrifugated at 8000 revolutions for 20 minutes, after which they were placed at 37 C. and tested at 3-, 9-, and 18-hour periods, the test dose being 3 c.c. It was expected that the high speed of centrifugation would remove all of the agar and, as a result, little or no poison would be formed. The tests which were made with the mixtures iced for 1 and 3 hours were strictly negative. Those made with mixtures which were iced for 6 and 9 hours caused acute death after incubation for 9 hours. Likewise, fatal shocks were obtained with the mixtures which had been iced for 12 and 24 hours, the tests being made after incubation for 18 hours. The mixtures which had been iced for 18 hours produced very severe shocks when tested at the 9- and 18-hour periods. It would seem that in the gel-serum mixtures iced for 6 hours or more a change was started similar to that at 37 C., but weaker. Still, the continued action of traces of agar is possible notwithstanding the high speed of centrifugation. Hence, the 4 deaths in 21 tests can be ascribed to either one of these causes.

TABLE 22

ACTION OF AGAR GEL (0 C.) ON GUINEA-PIG SERUM (0 C.) (RATIO 0.25:1. MIXTURE KEPT AT 37 C. THEN CENTRIFUGATED AND REINCUBATED)

Exper.	Guinea-Pig		Serum			Result
	No.	Weight	B. C. at 37 C.	A. C. at 37 C. (hr.)	c.c. (intravenously)	
I	1	195	—	2	3.0	Very slight
	2	220	—	5½	"	" "
II	3	176	2½′	2	"	" "
	4	218	2½′	5	"	5′35″
III	5	195	5′	2	"	Severe
	6	223	5′	5	"	Slight
IV	7	195	10′	2	"	2′40″
	8	195	10′	5	1.5	Very slight
V	9	201	15′	2	3.0	" "
	10	215	"	5	"	" "
VI	11	215	"	2	"	Very severe
	12	171	"	4	"	2′40″
VII	13	206	30′	—	"	Very slight
	14	212	"	2	"	3′55″
	15	195	"	5	2.0	Fair
VIII	16	185	1 hr.	—	3.0	3′5″
	17	222	"	2	2.0	Slight
	18	170	"	4	2.0	3′15″
	19	175	"	4	1.0	Very-near-kill
IX	20	175	2 hr.	—	3.0	3′55″
	21	185	"	2	3.0	3′40″
	22	185	"	5	2.0	3′15″
	23	170	"	5	1.0	Slight

By way of contrast with the foregoing, it is in order to present another series of experiments. The essential difference between the two sets lies in the fact that the gel-serum mixtures were placed directly at 37 C. for a variable time, after which they were centrifugated at 3000 revolutions for 10 minutes, and then the clear sera were again incubated for the periods stated in Table 22. It will be seen that the contact of agar and serum, at 37 C., before centrifugation, is a necessary factor to poison-production.

The series of 9 experiments given in Table 22 were made on 2 consecutive days. A pooled serum was used for Experiments 1 to 5, and another for Experiments 7 to 9. Experiment 6 was a duplicate of Experiment 5, but was made with a different serum.

It would seem from this series of experiments that incubation of the gel-serum mixture at 37 C. for 1 or 2 hours before centrifugation was the best treatment. It might be of interest to have made serial injections at intervals of 15 minutes with such material, and especially with such as had been kept at 37 C. for a longer time, 4 hours or more. Under such circumstances it might be possible to toxify the serum so that 1 c.c. would be a lethal dose. It will be seen from the table that 2 c.c. was fatal in Expers. 8 and 9, and that 1 c.c. produced a 'very-near-kill' in No. 19.

EXPERIMENTS WITH SOL-GEL AGAR

This method of treatment was first developed in connection with the work on rat serum, in which it proved to be superior to other procedures. Consequently, it was desirable to apply it to guinea-pig serum. Here also it gave exceptional results, as will be seen from Tables 24 and 26. The method undoubtedly offers the best possible conditions as regards the uniformity of a mixture, tho even here it must not be forgotten that the amount of shaking given a mixture influences the degree of sol division and hence the result.

The procedure consists first in liquefying the agar in a water bath at 100 C. for 15 minutes, after which it is placed in a Roux bath at 37 C. for 2 hours. In passing, it may be added that at this temperature the agar remains in the sol state for a day or more. It is then added to the pooled serum, previously warmed to 37 C., and the mixture is thoroughly shaken for 1 minute. This is then packed in cracked ice, usually for 2 or 3 hours, tho a shorter time is perhaps equally good. The finely subdivided agar is thus brought into a gel state, tho the mixture, depending on the amount of agar, may remain perfectly fluid. This finely dispersed gel on subsequent incubation rapidly induces the toxifying action in the ambient serum.

The toxicity of such mixtures was tested at given intervals. When the amount of agar is very small, the uncentrifugated fluid can be injected directly. With larger amounts of agar it is best to centrifugate a small portion. It has seemed on several occasions as if the presence of agar in the fluid injected modified the toxicity, rendering it less marked.

For most purposes the sol was measured out directly. When very small amounts were desired a 10-fold dilution with distilled water (37 C.) was made. This, on further dilution with 1 or 9 parts of water, gave dilutions such that 0.1 c.c. corresponded to 0.005 and 0.001 c.c. of sol, respectively. This insured a minimal dilution of the serum to be tested.

The volume of the guinea-pig serum used in each series of tests was not less than 10 c.c. and often from 20 to 30 c.c. A series of mixtures, each containing a definite amount of sol per cubic centimeter of serum, was tested. For convenience of reference these mixtures, designated by numbers, are presented in tabular form indicating the amounts of sol and of agar present in each, per cubic centimeter of serum, as well as the ratio of dry agar to serum.

TABLE 23

The Concentration of Different Agar-Serum Mixtures

Mixture	Sol (c.c. per c.c. of Serum)	Agar (mg.)	Agar-Serum Ratio 1:
1	0.0005	0.0025	400,000
1a	0.001	0.005	200,000
2	0.0025	0.0125	80,000
3	0.005	0.025	40,000
4	0.025	0.125	8,000
5	0.05	0.25	4,000
6	0.1	0.5	2,000
7	0.25	1.25	800
8	0.5	2.5	400
9	1.0	5.0	200

For the following tests the injection dose was placed at 1.5 c.c., or less, in order to get at the maximal poison-production. It is hardly necessary to state that with 3 c.c. the acutely fatal shock would have been more frequent. Indeed, it is possible, that with such dose even Mixture 2 might be found to toxify, and this would certainly be the case if the large dose (5 c.c.) of Bordet was used.

Mixture 1.—This mixture was kept in cracked ice for 3 hours, then at 37 C.; tests were made at intervals of 1½ hours up to 24 hours. In about one-half of these tests no effect was observable. In the others, slight excitability, deep respiration, and few jerky spasms were noted. In the tests made at 13½ and 19½ hours, dyspnea and spasms were well defined.

It will be shown that a corresponding mixture with rat serum, on incubation for 12 hours, killed in dose of 1 c.c. This fact indicates that even guinea-pig serum may show positive evidence of toxification provided larger doses be injected. Table 23 shows that this mixture represents an agar-serum ratio of 1 : 400,000.

Mixture 2.—This series of tests was made at the same time as the preceding, and under like conditions. The effects were about the same as those noted; the most marked results being at 1½, 13½, and 19½ hours.

Mixture 3.—One experiment similar to the preceding with tests at 1½-hour intervals up to 12 hours, gave very severe shocks (dyspnea, spasms, convulsions) at 4½, 7½, and 9 hours.

In another experiment, tests being made at half-hour intervals up to 3½ hours, a 'near-kill' was obtained in 1 hour; the other tests were relatively mild. In the belief that if the tests were made at quarter-hour intervals a fatal result would be obtained, another set was tested, in this way, up to 2 hours, but the effect in all was mild; possibly a more reactive serum would have given the desired result.

Additional tests were made with similar mixtures kept in ice for 1, 6, 9, and 14 hours, respectively, before incubation. In each of these experiments only 2 injections were made, at 12 and at 18 or 24 hours. Here also but mild effects were noted. In view of the variable resistance of guinea-pigs to the poison, occasional tests of this kind have very little meaning, when one is operating with minimal lethal doses; as pointed out, heretofore, the better procedure is to make the tests in duplicate, at frequent intervals.

It would appear from the foregoing that this mixture, which represents an agar-serum ratio of 1:40,000, is almost sufficient to toxify so that 1.5 c.c. will be fatal. In view of this, and of the further fact that rabbit serum is toxified by this mixture, there can be no doubt but that a dose of 3 c.c. would have given some fatal shocks. Since the agar-serum mixture of Bordet (1 + 5) has 1 mg. agar per cubic centimeter (1:1000), it will be seen that the one used in these tests contains but one-fortieth as much.

In a single trial of a corresponding gel-agar mixture, kept at 37 C. for 2 hours, Nathan obtained slight effects, the dose injected being 3.5 c.c. It is of interest by way of comparison, to note that Mixture 3 tested with rat serum, toxified it in 15 minutes so that 0.5 c.c. was fatal; while in 1 hour, 0.25 c.c. produced a like result.

Mixture 4.—Several of the experiments made with this mixture showed marked poison-production. Preliminary trials were made with mixtures iced for 1, 3, 6, 9, and 24 hours, respectively, before incubation. In each of these experiments only 2 tests were made, at 12 and at 18 or 24 hours. The results were slight or nil, except with the mixture which was iced for 6 hours. This, incubated for 12 hours, killed in dose of 1.5 c.c. and at the 24-hour test, in dose of 1 c.c., it gave a very severe shock or near-kill. This result led to a more extended series·of tests with another mixture iced at 0 C. for 6 hours; these, which are given in Table 25, show that, after incubation for 1¾ hours, a dose of 1.5 c.c. is fatal and that 1 c.c. is likewise at the 2-hour test.

Two additional experiments were made, the mixture in each case being iced for 3 hours. The first of these, incubated at 37 C. and tested at half-hour intervals up to 12 hours, was made at the same time as the first experiment described under Mixture 3; the same pooled serum was used for the two experiments. The tests made at 1½, 3, and 4½ hours gave very severe shocks; those at 6 and 10½ hours caused acute death; while at 12 hours, 1 c.c. produced a violent shock. The other tests were relatively mild. The second experiment, given in Table 24, shows that a dose of 1.5 c.c. was fatal after only 1 hour's incubation. The results of this experiment should be compared with the corresponding experiment with Mixture 5, made at the same time and with the same pooled serum.

TABLE 24

ACTION OF SOL-GEL ON GUINEA-PIG SERUM (MIXTURE 4, RATIO 0.025:1, KEPT AT 0 C. FOR 3 HR., THEN INCUBATED AND CENTRIFUGATED)

Guinea-Pig		Serum		Result
No.	Weight	B. C. at 37 C. (hr.)	c.c. (intravenously)	
1	180	½	1.5	Good shock
2	170	1	"	3′30″
3	183	1½	1.0	Very slight
4	171	2	"	Slight
5	202	2½	1.5	Very slight
6	175	3	"	3′30″
7	180	3½	"	Nil
8	185	5	"	3′40″

The results of two experiments with Mixture 4, iced for 3 and 6 hours, respectively, are given in Tables 24 and 25. It will be noted that the speed of poison-production under the conditions given, is fairly rapid. Further that it is possible to produce a 1-c.c. lethal dose with guinea-pig serum. The agar-serum ratio in this mixture is 1 : 8000, or one-eighth that employed by Bordet; and, moreover, the toxicity is increased 4- and even 5-fold, the dose employed by him being 4-5 c.c.

Mixture 5.—In general, the results obtained with this mixture were about the same as those with the preceding one. It was used not only for the experiments which immediately follow, but also for others to be described later (Tables 27, 28, 29, *et al.*). This and the next are probably the most useful mixtures.

Here as in the two preceding series, experiments were made with mixtures which had been iced for 1, 3, 6, 9, and 14 hours, respectively, before incubation. The first of these, kept at 0 C. for 1 hour, was incubated and tested at 3, 12, 18, and 24 hours. The first injection produced but slight effect, the second a severe shock, the third a near-kill, while the fourth had only moderate effect. Another mixture, likewise kept at 0 C. for 3 hours and then incubated, proved fatal when tested at 13 hours; retested at once in dose of 1 c.c. it caused a severe shock. This dose tried again at 18 hours caused a delayed death, in 7 hours.

TABLE 25

ACTION OF SOL-GEL ON GUINEA-PIG SERUM (MIXTURE 4, RATIO 0.025:1, KEPT AT 0. C. FOR 6 HR., THEN INCUBATED AND CENTRIFUGATED)

Guinea-Pig		Serum		Result
No.	Weight	B. C. at 37 C. (hr.)	c.c. (intravenously)	
1	200	½	1.5	Very slight
2	197	1	"	Nil
3	177	1½	"	Severe quiet shock
4	176	1¾	"	2′55″
5	170	2	1.0	3′55″
6	170	2¼	0.5	Very slight
7	175	2¼	1.0	Severe
8	195	2½	1.5	3′20″
9	180	3	"	3′45″
10	207	3½	"	Slight
11	206	4	"	Very slight
12	209	4½	"	Slight
13	180	5	"	Near-kill
14	195	5½	"	Slight
15	182	6	"	Severe
16	180	6½	"	Slight

In 3 other experiments in which the mixtures were kept at 0 C. for 6, 9, and 14 hours, respectively, and then incubated and tested at 12 and again at 18 or 24 hours, only slight effects were obtained. Apparently, prolonged icing is not as favorable for this mixture as is the shorter treatment.

Of three other mixtures, kept at 0 C. for 3 hours, one (designated No. 6) was tested after incubation for 1, 3, and 9 hours. The first injection resulted in a severe, the second in a fair, and the third in a very severe, shock. The experiment was repeated (No. 7) with another like mixture, the injections, however, being made at 1½-hour intervals up to 7½ hours. The injection at 1½ hours gave a fair shock, at 3 hours a near-kill, and at 4½ hours a typical acute death; the other tests were slight or nil. In the next attempt (No. 8), made with a like mixture, the tests were tried at half-hour intervals up to 3½ hours. This experiment was made at the same time and with the same pooled serum as was the experiment recorded in Table 24; the results were nearly alike in the two series, except that the effects with this mixture were less; the test at 1½ hours proved fatal, that at 3 hours gave a near-kill, while the others were slight or nil.

On reference to Tables 27 and 28 it will be seen that this mixture can render guinea-pig serum toxic in dose of 3 c.c. in 10 or 15 minutes.

Mixture 6.—Here, as in the preceding, a number of mixtures were tested after being kept at 0 C. for 3 hours. One of these tested after incubation for 1, 3, and 9 hours gave but slight effects compared with those of the corresponding experiment (No. 6) with Mixture 5, which was made at the same time and with the same pooled serum.

A second experiment was made at the same time and with the same pooled serum as in No. 7 of the preceding series. After incubation for 1½ hours the mixture killed in dose of 1.5 c.c., while 1 c.c. produced a severe shock. The same result was obtained at 3 hours, but at 4½ hours there was practically no effect.

In a third experiment in which the tests were made at intervals of 15 minutes up to 1½ hours, that at one-half hour killed, and those at 1 and 1½ hours produced severe shocks, while the other tests gave but slight effects.

In a fourth experiment, in which the mixture was kept under liquid paraffin, a very severe shock was obtained in 1 hour and a kill at 3 hours. Another mixture, after incubation for one-half hour, in dose of 3 c.c. caused acute death; retested at 1 hour, it killed in dose of 1.5 c.c.

Mixture 7.—Three experiments were made with this mixture. In each case it was kept at 0 C. for 3 hours, and then incubated. The first of these corresponded to the third of the preceding series and was made at the same time and with the same pooled serum. The test with 1.5 c.c. at 1 hour killed; with 1 c.c. a very severe shock was obtained at 1¼ hours, and at 1½ hours it killed.

In the second experiment, 1.5 c.c. was fatal in the test at one-half hour; at 1 hour, 1 c.c. caused a very severe shock, but had slight effect at 1½ hours. In a third experiment, the mixture after incubation was injected directly without previous centrifugation. The injections made at one-half hour intervals had little or no effect, until at 3 hours, when 1.5 c.c. caused typical death.

Mixture 8.—Three experiments with this mixture were made. In each the mixture was iced for 3 hours, then incubated, and at intervals 2.25 c.c. were removed and centrifugated; the resulting fluid, representing 1.5 c.c. of serum, was then injected. In one experiment (No. 1) 7 consecutive tests made during the first 2 hours, at 37 C., gave little or no effect. This result should be compared with that of the first experiment made with Mixture 9. The two series were made at the same time, side by side, with the same pooled serum. The failure in this case, if not due to a slight difference in the shaking of the mixtures, must be ascribed to individual variation in the animals tested. The experiment was therefore repeated, the mixture this time being given a more vigorous shaking for 1 minute; in this trial the test made at one-half hour caused typical death in 7 minutes, while 4 succeeding tests, up to 2 hours, gave but slight effects.

In a third experiment, the injection made after incubation for one-half hour was acutely fatal. The injection of 1 c.c. at the 1-hour period gave a very severe shock, but a like amount at 1½ and 2 hours had little effect.

It will be seen, therefore, that with this mixture also it is possible to toxify the serum so that 1.5 c.c. will kill. Nathan,[2] with corresponding amount of agar, failed to produce death with a 3-c.c. dose; this fact and the irregularities occurring with the ordinary mixture (0.2 + 1) led him to believe that an excess of agar was unfavorable. It is true that a mixture having much agar, at times does seem to give poor results, but that is probably not due merely to the presence of a large amount of agar. The experiments with this mixture and especially with the one that follows show that serum can be toxified under these conditions.

Mixture 9.—This mixture consisted of equal parts of sol and serum (Table 23). As in the preceding, the mixtures were iced for 3 hours and then incubated. At intervals 3 c.c. were removed and centrifugated; the resulting fluid, corresponding to 1.5 c.c. of serum, was then injected. In one experiment, corresponding to No. 1 with Mixture 8, and made at the same time and with the same pooled serum, the injection of 1.5 c.c. at ½- and 1½-hour periods caused death in 3 and 4 minutes, respectively, whereas the other 5 tests up to 2 hours were practically negative.

In a second trial which was made at the same time and with the same pooled serum as No. 2 with Mixture 8, the first test, made after

15 minutes at 37 C., gave a good shock; tests made at ½, ¾, 1, and 1½ hours showed little or no result, whereas at 2 hours 1.5 c.c. and also 1 c.c. produced typical acute fatal shocks.

Summation.—In the series of experiments with the 9 mixtures, as just outlined, the test dose only once exceeded 1.5 c.c.; occasionally, it was reduced to 1 c.c. On account of the smallness of the dose it is to be expected that the majority of the injections would yield little or no effect, and that the number of fatal shocks would be small. With such small doses the individual resistance of the animals is a factor in the scarcity of positive results.

Several important facts are to be noted. In the first place, it is seen that the toxicity appears to develop early and to persist for many hours. Further, that it is possible to produce in normal guinea-pig serum a toxicity such that 1.5 and even 1 c.c. are fatal. Also, that very minute amounts, as well as very large amounts of agar, may bring about the poison-production. Likewise that the sol-gel method is superior to the others in the speed and extent of the reaction induced. With very minute amounts of agar apparently a longer incubation is required than with larger quantities.

The results are collated in the following table:

TABLE 26

Summation of Results with the Series of Sol-Gel Mixtures; the Occurrence of Fatal Shocks with 1 and 1.5 c.c. of Treated Serum

Mixture	Number of Tests	Fatal Shocks with 1 c.c.	Fatal Shocks with 1.5 c.c.
1	17	0	0
2	17	0	0
3	36	0	0
4	46	1; at 2 hr.	9; at 1, 1¾, 2½, 3, 3, 5, 6, 10½, and 12½ hr.
5	30	1; at 18 hr.	3; at 1½, 4½, 13½ hr.
6	19	0	3; at ½, 1½, 3 hr.
7	28	1; at 1½ hr.	3; at ½, 1, 3 hr.
8	20	0	2; at ½, ½ hr.
9	14	1; at 2 hr.	3; at ½, 1½, 2 hr.

CENTRIFUGATION OF SOL-GEL MIXTURES

The effect of centrifugation at 8000 revolutions for 20 minutes on gel-serum mixtures which had been kept at 0 C. for varying periods from 1 to 24 hours, as regards poison-production has been briefly touched upon (p. 543). Inasmuch as only two or three injections, at long intervals, were made in those experiments, it was desirable to repeat the 3-hour one, employing, however, a sol-gel mixture, a longer centrifugation, and more frequent tests. The immediate object was to ascertain whether the mere contact of agar with serum, at 0 C., was sufficient to induce a change such that on subsequent incubation poison would be produced. To secure an unequivocal answer to a query of this kind, the agar must be completely removed from the serum, otherwise the presence of a mere trace, acting for a long time, may be sufficient to toxify. This removal of agar cannot be accomplished fully by centrifugation at 3000 revolutions, and for that reason such centrifugated sera do show poison-production on incubation. The only feasible way to effect a complete removal seemed to be by high-speed centrifugation for a very long time. Accordingly, a speed of 8000 revolutions for 60 minutes was resorted to.

The following experiment may be considered as a crucial test of the question as to the action of agar in mixtures iced for 3 hours, tho it was not made all in one day and with one pool of serum. On each of 3 consecutive days, a sol-gel mixture (No. 5) was prepared in the usual way, then immediately placed in cracked ice for 3 hours, after which it was centrifugated at the speed and for the time just mentioned. The perfectly clear pooled serum of each day was then transferred to test-tubes, which were placed in the Roux bath at 37 C. The injections were made at half-hour intervals up to and including the 10½-hour period. The injection dose contained the equivalent of 3 c.c. of serum. The total number of tests was 21, and of these Nos. 1 to 3 were made on the first day, Nos. 4 to 11 on the second, and Nos. 12 to 21 on the third.

The results of this experiment were practically nil; occasionally a few slight jerks, restlessness, but no dyspnea. It follows therefore that under these conditions, notwithstanding the large dose of serum and the risk of developing an autotoxic state by long incubation, there is no increase in toxicity beyond that which is present or developed in untreated normal serum. This would appear to be contrary to what might be expected if the reaction were purely one of adsorption, since after being iced for 3 hours the mixture might be expected to give some evidence of the latter.

The experiments hitherto given made it clear that, in order to secure a production of poison by the agar, heat must be applied before subjecting the mixture to centrifugation. This point established, the next step was to ascertain the effect of the complete removal of agar from a sol-gel mixture which had been kept at 0 C. for 3 hours and then incubated for a short time at 37 C.

For this purpose a sol-gel mixture (No. 5) was prepared in exactly the same manner as before, the amounts used being 2.5 c.c. of sol and 50 c.c. of guinea-pig serum. After thorough shaking for 1 minute the mixture was iced for 3 hours, and then placed at 37 C. for 10 minutes. The bulk of the mixture was now centrifugated at 8000 revolutions for 1 hour; a small portion was swung at the same time at 3000 revolutions for 6 minutes, and the equivalent of 3 c.c. of serum injected at once into Guinea-pig 1, producing a typical fatal shock. It is seen therefore that the mere incubation of the sol-gel mixture for 10 minutes was sufficient to render it toxic in dose of 3 c.c.; and further, that the bulk which was subjected to the high-speed centrifugation was initially toxic.

TABLE 27

CENTRIFUGATION AT 8000 R.P.M. APPLIED AFTER INCUBATION AT 37 C. FOR 10 MIN. SOL-GEL-MIXTURE 5, RATIO 0.05:1

Guinea-Pig		Serum			Result
No.	Weight	B. C. at 37 C. (min.)	A. C. at 37 C. (hr.)	c.c. (intravenously)	
1	212	10	—	3	3′10″
2	210	—	0	"	Fair shock
3	208	—	½	"	Very slight
4	195	—	1	"	Slight
5	190	—	1½	"	Nil
6	210	—	2	"	4′
7	200	—	2½	"	5′
8	198	—	3	"	Fair
9	175	—	3½	"	2′55″
10	185	—	4	"	Good
11	190	—	4½	"	4′10″

After centrifugation at 8000 revolutions, the clear serum was placed at 37 C. and tested at once, and at half-hour intervals, as indicated in Table 27. It will be seen that immediately after centrifugation the serum was apparently less poisonous than before; that after incubation for 2 hours the perfectly clear serum again became toxic, and that this state persisted, seemingly in wave form, till the end. This experiment should be compared with the corresponding one with rabbit serum, in which tests were made at quarter-hour intervals (Table 35). The results are expressed graphically in Chart 3. As pointed out heretofore, this and similar curves while conceivably due in part to variations in the amount of the poison must not be read wholly in that sense; for variation in the resistance of the animals must be considered, and probably this is the main if not sole cause of the wave character.

In the foregoing experiment the high-speed centrifugation was started at the time when the sol-gel mixture was toxic, that is, at what

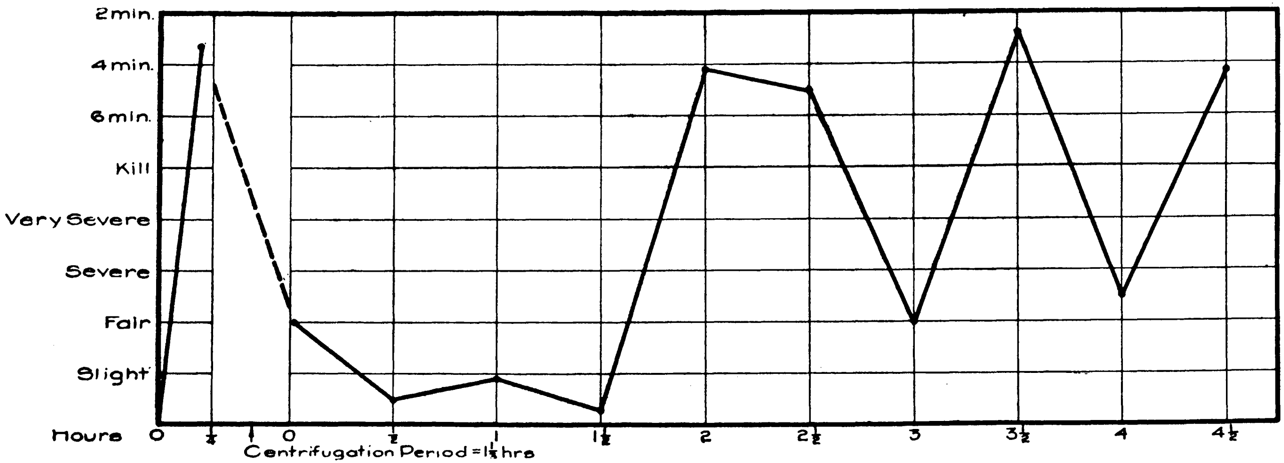

Chart 3. Centrifugation of the sol-gel mixture at 8000 r.p.m. after incubation. Apparent variation in toxicity of treated guinea-pig serum (Table 27).

might be called the crest of the toxic wave. It seemed that a different result might be obtained if the centrifugation was begun when the initial toxicity had dropped, and accordingly the experiment was repeated on the following day. The preparation of the sol-gel mixture and icing were exactly the same as before. The mixture was then placed at 37 C. and, at intervals as shown in Table 28, portions were removed, centrifugated at 3000 revolutions for 4 minutes and injected. It will be seen that incubation for 5 and 10 minutes seemed to produce but little poison, whereas incubation at 15 minutes gave rise to a fatal anaphylatoxin. The expected drop in toxicity did not occur until after incubation for 1 hour. At this point, the balance of the mixture was centrifugated at 8000 revolutions for 1 hour; the resulting clear serum was transferred to the Roux bath at 37 C., tests being made at once, and at half-hour intervals.

TABLE 28

I. Centrifugation at 8000 r.p.m. Applied at the First Drop in Toxicity
Sol-Gel-Mixture 5, Ratio 0.05:1

Guinea-Pig		Serum			Result
No.	Weight	B. C. at 37 C. (min.)	A. C. at 37 C. (hr.)	c.c. (intravenously)	
1	175	5	—	3	Slight
2	180	10	—	"	Slight
3	175	15	—	"	3′25″
4	170	30	—	"	3′30″
5	172	45	—	"	4′10″
6	204	60	—	"	Very slight
7	185	—	0	"	3′5″
8	200	—	½	"	Very severe
9	212	—	1	"	Very slight
10	185	—	1½	"	3′30″
11	200	—	2	"	4′
12	210	—	2½	"	2′50″
13	197	—	3	"	3′40″
14	212	—	3½	"	3′30″
15	198	—	4	"	3′10″

It will be seen from Table 28 and the corresponding chart (4), that the toxicity which seemed to have returned at the close of the centrifugation, was absent in the next 2 tests, after which it reappeared and remained in evidence until the end, giving rise to 6 consecutive kills. A similar toxic plateau is indicated in Chart 3, and also in Chart 5. It is to be expected that incubation of the sol-gel mixture for 1 hour would give a greater toxicity than incubation for only 10 minutes at 37 C., as in Table 27; and consequently a greater number of deaths. It may be questioned, however, whether this alone accounts for the peculiar curve.

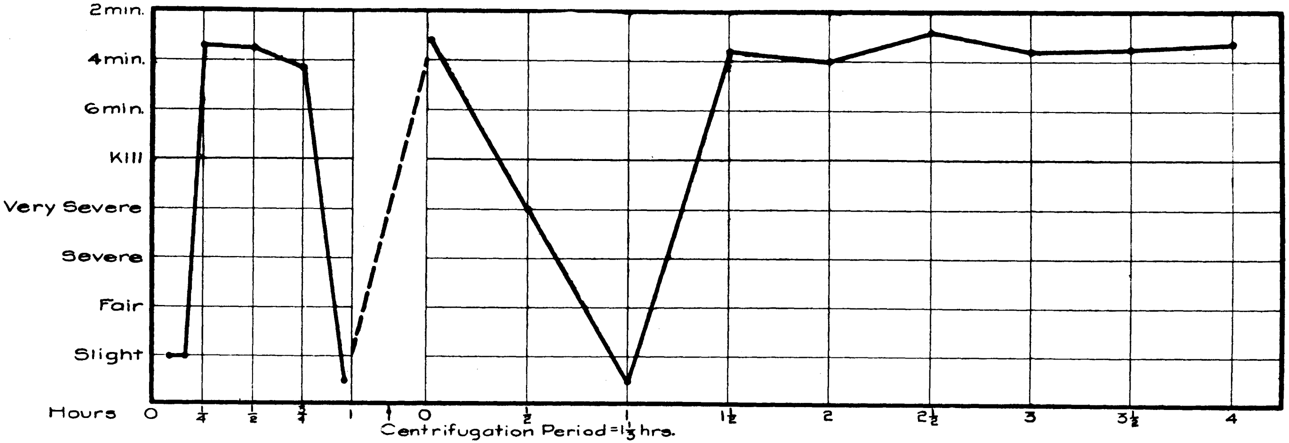

Chart 4. Centrifugation of the sol-gel mixture, at 8000 r.p.m.; after incubation till first drop in toxicity occurs. Apparent variation in toxicity of treated guinea-pig serum (Table 28).

The striking result obtained in the preceding experiment made its repetition desirable. This was done on the following day, the conditions being the same as before. In this trial the first kill was obtained at the 30-minute period, tho the 2 preceding tests gave severe shocks. The apparent drop in toxicity occurred at 1 hour, the same as before. The mixture was then centrifugated at 8000 revolutions for 1 hour, and the clear serum was put at 37 C., tests being made as usual at once and at half-hour intervals. Here again, after centrifugation, a rise in toxicity was seemingly obtained, followed in 1 hour by a marked decrease, and then a return to a plateau, which remained to the end, through 7 consecutive tests. The results of this experiment are given in Table 29, and again in Chart 5.

TABLE 29

II. Centrifugation at 8000 r.p.m. Applied at the First Drop in Toxicity
Sol-Gel-Mixture 5, Ratio 0.05:1

Guinea-Pig		Serum			Result
No.	Weight	B. C. at 37 C. (min.)	A. C. at 37 C. (hr.)	c.c. (intravenously)	
1	210	10	—	3	Severe shock
2	184	15	—	"	Very severe
3	194	30	—	"	3′10″
4	206	45	—	"	3′30″
5	208	60	—	"	Very slight
6	197	—	0	"	Severe
7	196	—	½	"	Very severe
8	209	—	1	"	Very slight
9	190	—	1½	"	4′
10	190	—	2	"	3′40″
11	187	—	2½	"	Near-kill
12	206	—	3	"	Very-near-kill
13	190	—	3½	"	2′50″
14	173	—	4	"	Very-near-kill
15	198	—	4½	"	3′30″

It is hardly to be expected that the same curve characteristics of toxicity should be met with in every experiment of this type. Apart from irregularities in technic, some variation must occur unless it be assumed that the pooled guinea-pig serum is chemically and physically a constant, and further that the animals are all equally susceptible. Thus, somewhat different results were encountered in an experiment intended to duplicate that given in Table 27. The pooled serum in this case was obtained from the blood of old female guinea-pigs, some of which weighed over 1 kilo. The sol-gel mixture 5, after being iced for 3 hours and incubated at 37 C. for 20 minutes, was centrifugated at 8000 revolutions for 1 hour. The serum was then tested at once and the balance, placed at 37 C., was tested at half-hour intervals (13 tests).

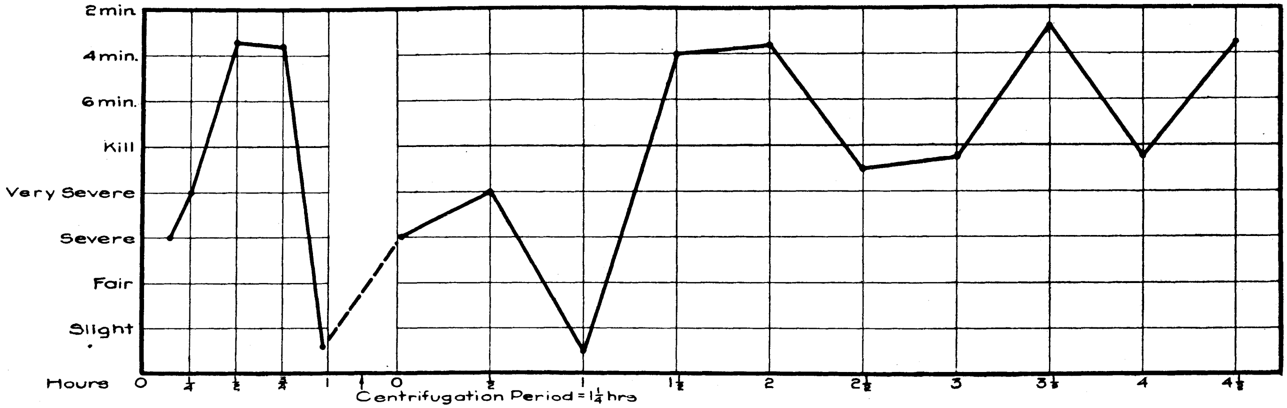

Chart 5, Conditions similar to those of Chart 4; see Table 29

The test which was made at once after centrifugation killed, as also did those made at ½, 1½, and 6 hours. Very severe shocks were obtained at the 2- and 4-hour periods, while the other tests had but slight effects. This series of tests if charted would show no indication of a plateau; the simplest meaning is that the amount of poison produced was less than in the other experiments and hence the individual resistance of the animals is more in evidence.

Summation.—These experiments with sol-gel Mixture 5, which had an agar-serum ratio of 1:4000, show that guinea-pig serum can be toxified in a dose of 3 c.c. on incubation for 10 minutes or more. The poison once produced remains in the serum for hours, even after high-speed centrifugation. Apparent oscillations in the poison content are seen, but these are to be interpreted as due to variations in the test animals. As in the case of trypanosomes, heat must be applied to the agar-serum mixtures. The failure of sera to become toxic after high-speed centrifugation of iced unheated mixtures indicates that adsorption plays no part in the production of poison. Further, ferment activity is not indicated, since there is no increase in the toxicity of incubated sera after high-speed centrifugation of agar-serum mixtures which have been iced and incubated for a short time.

ATTEMPTED DEMONSTRATION OF FERMENT ACTION

The possible presence of a ferment, in some way activated by the agar, was suggested here as in the previous work with trypanosomes. It seemed as if the hypothetical enzyme, once set free, should be demonstrable by means of serial dilutions into a pooled normal serum. On the other hand, if the poison existed preformed in the serum, and was merely uncovered by the removal of an antagonistic body, as supposed by Bordet[1] and others, then such solutions should show no increase in toxicity. A considerable number of experiments were made with rat and guinea-pig serum; those with the former will be considered later. The results at first were interpreted as indicative of a ferment action, but further study showed that such conclusions were not justifiable.

It will be of interest to present 2 such experiments, in which the conditions were practically identical. A pooled guinea-pig serum was employed for each series of tests, and the same agar was used for both. Three cubic centimeters of the 50 C. agar sol were added to 12 c.c. of the serum, previously warmed to 37 C.; the mixture, after thorough agitation for 1 minute, was at once centrifugated at 3000 revolutions for 5 minutes. The resulting fluid designated as A was divided into 3 portions: one was tested at once for its toxicity; the second was placed at 37 C. and tested at intervals as noted in the tables; the third was used to make dilutions. For the latter, 2 c.c. of A were added to 6 c.c. of pooled serum, giving a 1:4 dilution, or B. Diluting 2 c.c. of B in like manner gave the 1:16 dilution, or C, and this process was repeated yielding the 1:64 or D dilution, and the 1:256 or E dilution. These dilutions, in small Erlenmeyer flasks, were put in a Roux bath at 37 C. and tested at the times indicated.

TABLE 30

I. APPARENT FERMENT ACTION: PROGRESSIVE DILUTION OF 'TREATED' GUINEA-PIG SERUM WITH NORMAL SERUM

Series	Dilution	Guinea-Pig		Serum		Result
		No.	Weight	c.c.(intravenously)	Hours at 37 C.	
A	—	1	182	3	0*	6′30″
	—	2	195	3	4	3′15″
	—	3	185	2	4	Good shock
B	1:4	4	175	3	2	30′
	1:4	5	185	"	4	Good shock
C	1:16	6	190	"	4	Good shock
	1:16	7	201	"	23	45′
D	1:64	8	203	"	"	3′40″
E	1:256	9	180	"	"	4′20″
Control		10	202	"	"	9½ hr.

* This test was made 8 minutes after mixing the agar and serum; the mixture was not incubated.

The results of these tests are given in Tables 30 and 31. It will be seen in Table 30 that Serum A was acutely fatal at the initial test, which was made 8 minutes after mixing the sol and serum. It is to be noted that the control tests in both experiments with same serum, incubated for a long time but otherwise untreated, resulted in very delayed deaths. Similar delayed deaths occurred with dilutions C, D, and E of Table 31, and are to be interpreted as instances of primary or autotoxicity.

In the foregoing experiments the mixture was made with 50 C. sol and 37 C. serum, and consequently a favorable temperature for interaction was maintained for some minutes. Other tests, made under like conditions, gave equally good results. In fact in a number of experiments, the A and B portions were fatal when tested after incubation for 2 hours at 37 C. On the other hand, mixtures of iced gel and serum, kept in ice for ½, 1½, and 3 hours, and then centrifugated without any previous incubation at 37 C., yielded a serum which usually did not become toxic on subsequent incubation, and moreover failed to induce poison-production when diluted with normal guinea-pig serum.

In interpreting these results and those given in Tables 30 and 31, it is necessary to bear in mind that guinea-pig serum possesses an initial or primary toxicity which persists and may even increase on incubation. An even more important consideration is the fact that centrifugation at 3000 revolutions does not remove all the suspended agar. The presence of residual agar after such centrifugation can be easily shown by recentrifugating the apparently clear serum at 8000 revolutions, when a distinct coating of agar will be noted on the bottom of the glass.

TABLE 31

II. APPARENT FERMENT ACTION: PROGRESSIVE DILUTION OF 'TREATED' GUINEA-PIG SERUM WITH NORMAL SERUM

Series	Dilution	Guinea-Pig		Serum		Result
		No.	Weight	c.c. (intravenously)	Hours at 37 C.	
A	—	1	217	3	0	Slight
	—	2	197	"	6	Severe
	—	3	170	"	18	4 hr.
B	1:4	4	180	"	6	Good
	1:4	5	170	"	18	3′20″
C	1:16	6	177	"	6	Slight
	1:16	7	192	"	18	5 hr.
D	1:64	8	200	"	30	Good
	1:64	9	180	"	48	4 hr.
E	1:256	10	170	"	48	7½ hr.
Control		11	185	"	48	12 hr.

This residual agar is carried over into the successive dilutions, tho of course in decreasing amount, and the toxicity induced by such traces of agar, plus that which is primarily present, readily accounts for the observed results. This summation effect is most marked in the B dilution, for in 3 of 6 other tests acutely fatal sera were obtained in about 2 hours. When the B dilution was made into rat serum, the result was even more marked, for 0.5 c.c. of such would become acutely fatal. This phase of the subject will receive further attention in connection with rat serum in Part IV, where it will be shown that traces of agar react with the latter more readily than with guinea-pig serum.

The amount of agar which would be present in the dilutions A, B, C, D, and E, provided the original A mixture was not centrifugated, would correspond to 1 part of dry agar in 1000, 4000, 16,000, 64,000, 256,000 parts, respectively. The centrifugation of the mixture A obviously reduces the respective amounts of agar to a considerable extent. It has been pointed out heretofore that the sol-gel mixture 3, the agar-serum ratio of which is 1:40,000, will undoubtedly render guinea-pig serum toxic in dose of 3 c.c.; hence, even smaller amounts acting over a long time might be expected to have an action.

Ferment Adsorption.—In endeavoring to account for the action of the residual agar or trypanosomes, it might be assumed that such residues acted as adsorbing surfaces, and became laden with the ferment in much the same way that fibrin is supposed to take up thrombin. As a matter of fact, it has been shown by Hamburger[8] that agar does adsorb digestive ferments. If something similar occurred in the course of anaphylatoxin-production, it should be possible to demonstrate the presence of an adsorbed enzyme, either by using such agar directly, or its extract, as a means of inducing toxicity.

A number of experiments were made with the object of detecting an adsorbed ferment. For this purpose, the precipitate obtained on centrifugation of the incubated agar-serum mixture was taken up in guinea-pig serum, previously inactivated at 56 C. for half an hour, and thoroughly mixed with the aid of a finely drawn-out pipet. Such mixtures were then incubated for varying lengths of time and tested. At the same time control mixtures of agar and distilled water were carried through exactly the same steps. Repeatedly, slight or fair shocks were obtained with the inactivated serum which had been treated with the supposedly ferment-laden agar, whereas the effect in the control tests was practically nil. The injection dose in these tests was 3 c.c. It seemed, therefore, as if the treated agar was able slightly to toxify the inactivated serum. The results, however, were by no means conclusive, and the slight effects could easily be ascribed to the more facile redistribution of the laden agar through the heated serum; in the controls the clean agar was not so easily dispersed.

These experiments were made on the assumption that the inactivation consisted in destroying the enzyme, the matrix being unaffected. In the light of subsequent work, it is quite likely that a change in the matrix does occur. If a laden agar such as was used in the experiments is added to normal guinea-pig serum, and the mixture tested at intervals of 15 minutes, it will be found to toxify, but the speed is not increased over that with untreated agar.

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[8] Arch. néerl. d. sc. exact. et nat., 1908, 13, p. 428.

Another effort at demonstrating the presence of a ferment in the treated agar consisted in testing the action of aqueous extracts of the latter. The agar deposit obtained on centrifugation of an incubated sol-gel serum mixture was thoroughly rubbed up with distilled water, and this suspension was digested at 45 C. for 1 hour in the hope of dissolving out any adsorbed ferment. It was then centrifugated and the clear water extract was added to 1-1½ volumes of normal guinea-pig serum and incubated at 37 C. for 2, 3, and even 5 hours. The equivalent of 3 c.c. serum was injected at half-hour intervals. In 3 of these experiments, the results were practically nil; in a fourth, some dyspnea and moderate spasms were obtained in the tests made at 4 and 5 hours. This latter slight result was obtained with an extract of a deposit from a mixture which had been centrifugated at 8000 revolutions for 1 hour, in order to remove the agar as completely as possible; the extract was incubated with an equal volume of serum. The effects produced by the injection of 6 c.c. of this dilute serum are consequently those which could be expected from the incubation of diluted normal serum. All these attempts to prepare an active extract from the laden agar must be considered as negative.

Taken as a whole, the dilution experiments, as well as those with laden agar and with extracts of such, fail to give any clear or definite evidence of the participation of a ferment in anaphylatoxin-production in guinea-pig serum.

SUMMARY

The production of anaphylatoxin in guinea-pig serum through the aid of agar is a reaction of great speed. With proper conditions, incubation for from 8 to 10 minutes at 37 C. may render the serum fatal in dose of 3 c.c.

It is possible to reduce the lethal dose to 1 c.c.

The poison-production rapidly reaches a maximum, which appears to persist for a considerable time at 37 C.

The toxicity of such serum, when tested at regular intervals, appears to show more or less oscillation, as in the case of the trypanosome anaphylatoxin. Such variations are not due to a change in the amount of the poison, but to a varying resistance of the test animals.

The physical state of the agar is an important factor in the production of the poison. The 50 C. sol can be used to toxify serum, but the

0 C. gel apparently gives better results. The best results are obtained by the sol-gel method.

With the sol-gel method, a mixture (No. 3) representing 0.025 mg. of agar per cubic centrimeter of serum, or 1:40,000, when incubated for an hour or more, became so toxic that 1.5 c.c. nearly proved fatal. This represents one-fortieth the amount of agar and one-third the dose employed by Bordet.

The most convenient mixtures are probably Nos. 4 and 5, with 0.025 and 0.05 c.c. of agar sol, respectively, per cubic centimeter of serum.

The reaction is not inhibited by an excess of agar, since a 1:1 mixture (No. 9) may render a serum toxic in dose of 1 c.c.

Some incubation of the agar-serum mixtures at about 37 C. is necessary. Mere contact at 0 C., even for many hours, does not result in poison-production—a fact which contraindicates adsorption as a factor in the reaction. Mixtures containing very small amounts of agar require a longer period of incubation than others.

The individual sera vary somewhat in the ease with which they can be toxified. This is not due to the presence of lipoidal matter, or to the age of guinea-pigs. The kind of food as in the case of rabbits may be a factor.

The poison persists in the treated serum after the complete removal of the agar by centrifugation at 8000 revolutions. It is not destroyed by heating such serum to 60 C. for half an hour.

No evidence was found to show that this anaphylatoxin was either preformed, or the result of ferment action. On the contrary, the findings oppose both views.